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Veterinary Parasitology 129 (2005) 149–158
veterinary parasitology
www.elsevier.com/locate/vetpar

Association of the *kdr* and *superkdr* sodium channel mutations with resistance to pyrethroids in Louisiana populations of the horn fly, *Haematobia irritans irritans* (L.)

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Accepted 12 January 2005

Abstract

Pyrethroid resistance in three horn fly populations in Louisiana was monitored by weekly fly counts, filter paper bioassays, and diagnostic PCR assays for the presence of pyrethroid resistance-associated mutations in the sodium channel gene coding region. The PCR assay for the knockdown resistance (*kdr*) and *superkdr* sodium channel mutations was used to determine the frequency of the target site insensitivity mechanism in the populations of horn flies, which possessed varying degrees of insecticide resistance. The bioassays and frequency of homozygous-resistant (RR) *kdr* genotypes were relative predictors of the fly control subsequently observed. Flies exposed to filter paper impregnated with a discriminating concentration of one of four different insecticides were collected when 50% mortality was estimated. Genotypes for the dead flies and the survivors were determined by the PCR assay. The results of the PCR assays indicated that the genotype at the *kdr* locus of the flies exposed to the two pyrethroids had an effect upon whether the flies were considered to be alive or dead at the time of collection. The *kdr* genotype of flies exposed to either diazinon or doramectin was unrelated to whether the flies were considered to be alive or dead, except for a single comparison of flies exposed to diazinon. When possible interactions of the *kdr* and *superkdr* mutations were compared, we found that there were no associations with the response to diazinon and doramectin. For one location, there were no survivors of the 75 flies with the SS–SS (*superkdr–kdr*) homozygous susceptible wild type genotype exposed to pyrethroids, while there were survivors in all of the other five genotypes. The SS–RR genotype flies were more susceptible to the pyrethroids than the SR–RR flies, but that was not the case for exposure to diazinon or doramectin. In the St. Joseph population, there were an adequate number of flies to demonstrate that the SS–SR genotype was more susceptible to pyrethroids than the SS–RR and that flies with the SR–SR genotype were more susceptible to pyrethroids than the flies with the SR–RR genotype.

Keywords: PCR; Resistance mechanism; Sodium channel; Mutation; *Haematobia irritans* (L.)

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doi:10.1016/j.vetpar.2005.01.007

1. Introduction

The horn fly, *Haematobia irritans irritans* (L.), is the primary economic pest of beef cattle in the United States, costing producers with pastured cattle an estimated US$ 876 million annually (Kunz et al., 1991). Horn fly control primarily has been based on the use of insecticides, and this control strategy has led to resistance to most commercially available products (Byford et al., 1985; Sparks et al., 1985). Currently, the majority of the products used for horn fly control are either pyrethroids or organophosphates (OPs).

When pyrethroid-impregnated ear tags were first introduced, treatment with the tags often provided season-long control of horn flies (Ahrens and Cocke, 1979). However, within 2–3 years of continuous use of these tags, pyrethroid-resistant horn fly populations were identified (Quisenberry et al., 1984). Since that time, pyrethroid-resistant horn flies have been reported in most areas of the United States. Since resistance to one pyrethroid often confers cross-resistance to other insecticides in the pyrethroid class (Byford et al., 1985), the utility of this class of pesticide for horn fly control has been greatly diminished.

The physiological and biochemical mechanisms associated with pyrethroid resistance in the horn fly include reduced target site sensitivity, reduced penetration and increased metabolism or detoxification (Byford et al., 1985; Sparks et al., 1990). Bull et al. (1988) provided direct evidence that enhanced metabolic detoxification can contribute to pyrethroid resistance in horn flies, but the authors acknowledged that target site insensitivity is the major factor in pyrethroid resistance in the horn fly. The target site insensitivity resistance mechanism is commonly referred to as knockdown resistance (*kdr*). Guerrero et al. (1997) identified two mutations (designated *kdr* and *superkdr*) in the horn fly sodium channel gene that are associated with pyrethroid target site resistance. Presence of identical sodium channel mutations has been correlated with pyrethroid resistance in other insects, including *Drosophila melanogaster* (Meigen) (Amichot et al., 1992), *Blattella germanica* (L.) (Miyazaki et al., 1996), and *Musca domestica* L. (Knipple et al., 1994). Further work led to the development of a PCR-based assay to allow the detection of the presence of these two mutations in individual horn flies (Jamroz et al., 1998).

Jamroz et al. (1998) compared traditional bioassay-derived resistance ratios to the allelic frequency of the mutations and found a clear correlation in the laboratory colonies, but not for field populations. In their study, a resistance ratio of 17 for cyhalothrin was associated with *kdr* in a laboratory colony, but a resistance ratio of 18 for a field population was not associated with *kdr*, and attributed to metabolic resistance. The purpose of this study was to use the PCR assay to verify the target site resistance mechanism in various field populations of horn flies in Louisiana and to determine if sodium channel genotype was associated with survival of discriminating doses of different insecticides or could be predictive of fly control in the field.

2. Materials and methods

The bioassays were conducted at three Louisiana State University Agricultural Center research stations, prior to the treatment of the cattle with two Saber Extra® ear tags (10% λ-cyhalothrin + 13% piperonyl butoxide, Schering-Plough, Kenilworth, NJ). The locations were the Red River Research Station, Bossier City, the Macon Ridge Research Station, Winnsboro, and the Northeast Research Station, St. Joseph, LA. At each location, the number of horn flies per side on 10 randomly selected cattle was estimated once per week. Fly counts began at least 2 weeks before tags were administered, and were continued until the average number of flies was above 50 flies per side. The counts were made before 8:00 a.m. by one individual with the aid of binoculars.

2.1. Bioassays for LC_{50}'s

Horn flies were collected from cattle by the use of aerial nets and tested for susceptibility using the impregnated filter paper method (Sheppard and Hinkle, 1987). Technical grade insecticides used were the OP, diazinon, and the pyrethroids, λ-cyhalothrin and permethrin. Fly mortality was determined after a 4 h exposure period; flies unable to walk were considered dead. Three replicates of approximately 25 flies each were used for each insecticide concentration. Flies obtained from the colony maintained at the Knipling–Bushland U.S. Livestock Insects Research Laboratory,

USDA-ARS, Kerrville, TX, were used as the reference susceptible strain. Data were analyzed by probit analysis using POLO-PC (LeOra Software, 1987). An LC_{50} (expressed in $\mu g/cm^2$) for the three insecticides was obtained for the reference strain and field collected flies. The levels of resistance, subsequently referred to as resistance factors (RF), were calculated by dividing the LC_{50} from field populations by the LC_{50} from the Kerrville reference strain.

2.2. Discriminating concentrations of insecticides

In assays to correlate PCR and survival of a filter paper discriminating concentration exposure, four insecticides were used at one concentration with three replicates. Filter papers impregnated with permethrin, λ-cyhalothrin, diazinon, and doramectin at 400, 160, 13.8, and 78.6 $\mu g/cm^2$, respectively, were used. The doramectin treatment was 1 ml of Dectomax® pour-on (Pfizer Animal Health, NY; 5 mg doramectin per ml). The filter papers were put into disposable petri dishes and approximately 50 flies were placed in each dish. When approximately half of the flies were dead, the petri dishes were placed in a cage and living flies in one vial and dead flies in a separate vial. The vials were placed on dry ice, transported to the laboratory, and then stored at −80 °C for PCR.

2.3. PCR technique

The sodium channel genotype of horn flies was determined using the PCR technique previously described in detail (Guerrero et al., 2002). The genomic DNA was isolated from individual frozen adult flies following a protocol reported by Guerrero et al. (1998). PCR was performed by the procedure of Jamroz et al. (1998), using 20 μl reactions optimized for primer annealing temperature and sequence and $MgCl_2$ concentration to allow for the consistent discrimination between the susceptible (S) and resistant (R) sodium channel *kdr* and *superkdr* alleles. Final optimized reaction conditions used 2.5 μl of diluted genomic DNA solution from a single fly (25–150 ng), primers as described below, 14 mM Tris[(hydroxymethyl)aminomethane hydrochloride] (pH = 8.3), 70 mM KCl, 0.15 mM each dNTP, 4.5 mM $MgCl_2$, and 0.2 μl of a 1:1 (v:v) mix of *AmpliTaq* DNA polymerase (5 units/μl stock; Perkin-Elmer, Foster City, CA) and TaqStart antibody (1.1 μg/μl stock; Clontech, Palo Alto, CA). Primers were utilized at the following concentrations: R1, R2, R3, FG-130 and FG-134 = 1.5 μM; R5 and R6 = 1.75 μM; and R4 = 0.3 μM (Table 1). Each complete PCR pyrethroid resistance genotyping assay required two amplification reactions. To assay for the presence of susceptible alleles, only primers R1–5 and FG-130 were included in the reaction mix. To assay for resistant alleles, only primers R1–4, R6, and FG-134 were included in the reaction mix. Amplification was carried out using a DNA Engine (MJ Research, Watertown, MA) and reaction products were fractionated on 3.5% NuSieve agarose (FMC BioProducts, Rockland, ME) TBE gels and DNA was visualized by staining with GelStar DNA Staining Dye (FMC Bioproducts, Rockland, ME) and UV illumination.

Table 1
Sequences of PCR primers

Primer ID	Sequence	Description	Annealing site[a]
FG-130	5′-TACTGTTGTCATCGGCAATC-3′	Sus forward *kdr* diagnostic[b]	nt #429–448
FG-134	5′-TACTGTTGTCATCGGCAATT-3′	Res forward *kdr* diagnostic[c]	nt #429–448
R1	5′-GACAAATTCAAAGATCATGAAT-3′	Forward control primer	nt #274–295
R2	5′-TACGTTTCACCCAGTTCTTA-3′	Reverse *kdr* control primer	nt #573–592
R3	5′-TCGTGTATTCAAATTGGCAAA-3′	Forward *superkdr* control primer	nt #105–125
R4	5′-CGAAAAGTTGCATTCCCATA-3′	Reverse control primer	nt #231–250
R5	5′-ACCCATTGTCCGGCCCA-3′	Sus reverse *superkdr* diagnostic[b]	nt #161–177
R6	5′-ACCCATTGTCCGGCCCG-3′	Res reverse *superkdr* diagnostic[c]	nt #161–177

[a] Numbering is 5′–3′ on sense strand of horn fly sodium channel fragment (Guerrero et al., 1997).
[b] Susceptible horn fly sodium channel cDNA fragment Genbank accession no. U83871: nt #161–177 5′-TGGGCCGGACAATGGGT-3′, nt #429-448 5′-TACTGTTGTCATCGGCAATC-3′.
[c] Resistant horn fly sodium channel cDNA fragment Genbank accession no. U83873: nt #161–177 5′-CGGGCCGGACAATGGGT-3′, nt #429-448 5′-TACTGTTGTCATCGGCAATT-3′.

Table 2
Horn fly control and bioassay results for each location of the study

Location		Weeks of λ-cyhalothrin control[a]		Permethrin		Diazinon	
		LC_{50} (95% F.L.)[b]	RF[c]	LC_{50} (95% F.L.)	RF	LC_{50} (95% F.L.)	RF
Red River	8	5.9 (3.5–9.8)	25.6	3.8 (1.93–7.0)	3.7	2.0 (0.91–4.6)	3.1
Winnsboro	0	127.0 (87.4–256.3)	554.5	27.0 (20.3–35.6)	26.0	1.2 (1.05–1.4)	1.9
St. Joseph	3	34.4 (21.1–65.1)	150.4	10.6 (8.6–13.1)	10.2	0.8 (0.6–1.0)	1.25

[a] An average of fewer than 50 horn flies per side.
[b] Microgram active ingredient/cm^2 (95% fiducial limits).
[c] Resistance factor (RF) = test LC_{50}/Kerrville reference susceptible colony LC_{50}.

Table 3
Minutes to estimated 50% mortality of horn flies exposed to treated filter papers

Location	Permethrin (400 μg/cm^2)	λ-Cyhalothrin (160 μg/cm^2)	Diazinon (13.8 μg/cm^2)	Doramectin (78.6 μg/cm^2)
Red River	12	12	17	66
Winnsboro	35	40	22	47
St. Joseph	17	17	14	60

Comparisons of the survival of different genotypes and exposure to the different insecticides were made as a contingency table using Fisher's exact test, when there were at least 10 flies for each of the two genotypes selected for comparison.

3. Results

3.1. Bioassays

Data from the bioassays for susceptibility of the tested flies prior to administering the ear tags, combined with the subsequent number of weeks of control provided by the ear tags, indicated that there was pyrethroid resistance at each location (Table 2). The combined data also suggested that RF values could be used as relative predictors of the efficacy of the pyrethroid ear tag treatments of the cattle. That is, the highest RFs for the two tested pyrethroids were measured at Winnsboro, where no subsequent control was obtained, and the lowest RFs for the pyrethroids were measured at Red River, where 8 weeks of control was observed. Similarly, two other measures were reflective of the level of resistance in the fly population at each location: (1) the time to reach 50% mortality upon exposure to the two pyrethroids (Table 3) and (2) the frequency of the homozygous-resistant (RR) *kdr* genotype (Table 4). The frequency of the *superkdr* homozygous-resistant genotype did not appear to be correlated with the other measures of resistance (Table 4).

3.2. Discriminating concentration versus survival of different genotypes

It was of interest to evaluate if the effect of resistant (R) allele dosage at each locus (*superkdr* and *kdr*) would reveal trends in survival of horn flies. R allele dosage at the *kdr* locus had a positive effect on the horn fly's survival of pyrethroid exposure. There was a higher probability that the flies with one R *kdr* allele (SR *kdr* genotype) survived exposure to pyrethroids when compared to homozygous susceptible (SS *kdr* genotype) flies, and flies with two R *kdr* alleles (homozygous-resistant (RR) *kdr* genotype) were more likely to survive than those with one R *kdr* allele (SR *kdr* genotype; Tables 5–7). The R allele gene dosage at both the *kdr* and *superkdr* loci had no effect on survivorship in the diazinon or doramectin exposure

Table 4
Frequency of homozygous-resistant flies at the three study sites

Location	No. of flies	% RR flies	
		superkdr locus	*kdr* locus
Red River	376	2.4	10.1
Winnsboro	219	4.1	72.2
St. Joseph	224	4.0	52.2

Table 5
The *kdr* genotype of horn flies that were either dead or alive at the time when 50% mortality was estimated at Red River

Treatment	Alive			Dead			*P*-value[a]	
	SS	SR	RR	SS	SR	RR	SS vs. SR	SR vs. RR
Permethrin	0	7	9	34	39	0	0.019	<0.001[b]
λ-Cyhalothrin	0	5	12	41	42	0	0.058	<0.001
Diazinon	16	25	9	21	30	1	1.000	0.014
Doramectin	33	31	6	8	6	1	0.774	1.000[b]

[a] Fisher's exact test two-sided.
[b] Fewer than 10 flies in RR genotype.

Table 6
The *kdr* genotype of horn flies that were either dead or alive at the time when 50% mortality was estimated at St. Joseph

Treatment	Alive			Dead			*P*-value[a]	
	SS	SR	RR	SS	SR	RR	SS vs. SR	SR vs. RR
Permethrin	0	2	26	2	24	2	ND[b]	<0.001
λ-Cyhalothrin	0	2	26	1	20	7	ND	<0.001
Diazinon	2	9	17	5	12	11	ND	0.257
Doramectin	2	10	16	2	14	12	ND	0.404

[a] Fisher's exact test two-sided.
[b] ND, not determined, since fewer than 10 flies in genotype group.

assays except for a single comparison of flies exposed to diazinon at Red River (Table 5).

As in the previous studies of Jamroz et al. (1998) and Guerrero et al. (2002), we did not find the *superkdr* mutation in the absence of a *kdr* mutation; that is, the SR–SS, RR–SS and RR–SR (*superkdr–kdr*) genotypes were not found (Table 8). At Red River, over 40% of the flies tested were SS–SS (Table 8). These flies died rapidly following pyrethroid exposure and the survivors and dead flies were collected at 12 min (Table 3). There were no SS–SS survivors after 12 min; compared to all other genotypes, the SS–SS individuals were significantly more susceptible to pyrethroids than flies with resistant *kdr* or *kdr* plus *superkdr* genotypes (Table 9). While there were no survivors of the 75 Red River flies with the SS–SS genotype exposed to pyrethroids, there were survivors in all of the other five genotypes. The SS–SS genotype flies did not respond differently than the other genotypes when exposed to diazinon or doramectin (Tables 5–7).

At St. Joseph and Winnsboro, there were not enough SS–SS flies to allow statistical comparison for that genotype. At Winnsboro, there were adequate numbers to examine the influence of the R allele gene dosage at the *superkdr* locus on flies with the RR genotype at the *kdr* locus (Table 9). The SS–RR genotype flies were more susceptible to the pyrethroids than the SR–RR flies, but that was not the case for exposure to diazinon or doramectin. This comparison was not possible at St. Joseph because

Table 7
The *kdr* genotype of horn flies that were either dead or alive at the time when 50% mortality was estimated at Winnsboro

Treatment	Alive			Dead			*P*-value[a]	
	SS	SR	RR	SS	SR	RR	SS vs. SR	SR vs. RR
Permethrin	0	2	26	0	9	20	ND[b]	0.041
λ-Cyhalothrin	0	4	22	0	11	17	ND	0.070
Diazinon	0	6	20	1	6	13	ND	0.734
Doramectin	1	7	20	0	8	20	ND	1.000

[a] Fisher's exact test two-sided.
[b] ND, not determined, since fewer than 10 flies in genotype group.

Table 8
Genotypes of horn fly populations at each location

Location	Total no. of flies	Genotype: (*superkdr–kdr*)[a]						S allele frequency (%)[b]
		SS–SS	SS–SR	SS–RR	SR–SR	SR–RR	RR–RR	
Red River								
No. of flies	376	153	81	5	104	24	9	
Total (%)		40.69	21.54	1.33	27.66	6.38	2.39	65.29
Winnsboro								
No. of flies	219	2	31	64	28	85	9	
Total (%)		0.91	14.16	29.22	12.79	38.81	4.11	14.38
St. Joseph								
No. of flies	224	14	61	52	32	56	9	
Total (%)		6.25	27.23	23.21	14.29	25	4.02	27.01

[a] S denotes a susceptible allele, R denotes a resistant allele with *superkdr* genotype indicated on the left of hyphen and *kdr* genotype on right.
[b] S allele frequency (%) = [No. of S alleles/(No. of S alleles + No. of R alleles)]×100.

Table 9
The *superkdr–kdr* genotype of horn flies that were either dead or alive at the time when 50% mortality was estimated at the three study sites

Location	Treatment		Genotype[a]						*P*-value[b]
			SS–SS	SS–SR	SS–RR	SR–SR	SR–RR	RR–RR	
Red River									
	Permethrin	Alive	0	4	1	3	5	3	0.0003[d]
		Dead	34	23	0	16	0	0	
	λ-Cyhalothrin	Alive	0	1	1	4	7	4	<0.0001[d]
		Dead	41	11	0	31	0	0	
	Diazinon	Alive	16	10	3	15	6	0	0.4154[d]
		Dead	21	17	0	13	0	0	
	Doramectin	Alive	33	12	0	19	4	2	0.5663[d]
		Dead	8	23	0	3	1	0	
Winnsboro									
	Permethrin	Alive	0	0	5	2	18	3	0.0059[e]
		Dead	0	5	13	4	7	0	
	λ-Cyhalothrin	Alive	0	1	6	3	14	2	0.0217[e]
		Dead	0	5	12	6	5	0	
	Diazinon	Alive	0	4	9	2	11	0	0.4813[e]
		Dead	1	6	8	6	5	0	
	Doramectin	Alive	1	4	8	3	11	1	0.156[e]
		Dead	0	6	3	2	14	3	
St. Joseph									
	Permethrin	Alive	0	0	13	2	12	1	<0.001[f]
		Dead	2	16	1	8	1	0	
	λ-Cyhalothrin	Alive	0	2	8	0	5	3	0.020[f]
		Dead	1	16	7	4	15	0	
	Diazinon	Alive	2	8	7	1	10	0	1.0[f]
		Dead	5	7	5	5	5	1	
	Doramectin	Alive	2	5	7	5	7	2	0.414[f]
		Dead	2	7	4	7	6	2	

Table 9 (*Continued*)

Location	Treatment		Genotype[a]						*P*-value[b]
			SS–SS	SS–SR	SS–RR	SR–SR	SR–RR	RR–RR	
	Permethrin	Alive	0	0	13	2	12	1	<0.001[g]
		Dead	2	16	1	8	1	0	
	λ-Cyhalothrin	Alive	0	2	8	0	5	3	<0.001[c,g]
		Dead	1	16	7	4	15	0	
	Diazinon	Alive	2	8	7	1	10	0	0.064[c,g]
		Dead	5	7	5	5	5	1	
	Doramectin	Alive	2	5	7	5	7	2	0.695[g]
		Dead	2	7	4	7	6	2	

[a] S denotes a susceptible allele, R denotes a resistant allele with *superkdr* genotype indicated on the left of hyphen and *kdr*, genotype on right.
[b] Fisher's exact test two-sided.
[c] Fewer than 10 flies per genotype.
[d] SS–SS vs. all others.
[e] SS–RR vs. SR–RR.
[f] SS–SR vs. SS–RR.
[g] SR–SR vs. SR–RR.

both the SS–RR and SR–RR genotypes were in the permethrin alive group (i.e. the flies were more resistant at Winnsboro). At St. Joseph, there were adequate numbers of flies to demonstrate that the SS–SR genotype was more susceptible to pyrethroids than the SS–RR and that flies with the SR–SR genotype were more susceptible to pyrethroids than the flies with the SR–RR genotype (Table 9).

4. Discussion

The traditional filter paper bioassays, particularly those for λ-cyhalothrin, were good indicators of the relative susceptibility of the fly populations to pyrethroids as was subsequently demonstrated by the number of weeks of control observed for the λ-cyhalothrin tag treatments. The RF of 25.6 for λ-cyhalothrin for the flies tested at the Red River Station was similar to the 17–18 range for cyhalothrin that was shown by Jamroz et al. (1998) to be associated with the *kdr* mutation in a laboratory-resistant population (66% resistant *kdr* allele frequency), but not in a field collected population (37% resistant *kdr* allele frequency). At Red River, there was a 34.7% resistant *kdr* allele frequency similar to the field population tested by Jamroz et al. (1998).

An objective of this study was to determine if different genotypes responded differentially to pyrethroids and other insecticides. We assumed that unless metabolic resistance mechanisms exist in the fly and are linked to the sodium channel mutations, then we should be able to ask questions about survival versus specific sodium channel genotype comparisons. There was a higher probability that the flies with resistant *kdr* alleles were alive after exposure to pyrethroids when compared to flies without the resistant alleles, and this would be expected if the mechanism of resistance measured by the PCR assay was one of the resistance mechanisms contributing to the result of the bioassay. For example, at Red River, there were few homozygous-resistant (RR) *kdr* individuals, and all of those flies survived the exposure to pyrethroids helping to establish the role of this genotype in pyrethroid resistance. Previously, there has been speculation regarding the role that *kdr* and *superkdr* mutations play in pyrethroid resistance; Williamson et al. (1996) and Jamroz et al. (1998) concluded that the *kdr* mutation does confer significant levels of pyrethroid resistance on the horn fly. Our study helps to confirm this conclusion. Jamroz et al. (1998) compared the allelic frequencies of populations to RFs, while our study offers a direct comparison of the differential survival of flies with different genotypes exposed to the same insecticide.

Speculations have been made about possible negative or positive cross-resistance of pyrethroid-resistant flies to other insecticides, particularly regard-

ing potential negative cross-resistance for OPs (Sheppard and Marchiondo, 1987). In every comparison, we demonstrated that pyrethroid treatments influenced the response of flies relative to the *kdr* genotype and the other treatments did not. The one exception was for the SR versus RR *kdr* genotypes exposed to diazinon at Red River, and those results suggest a positive rather than a negative cross-resistance.

The results of our assays indicated that there is not cross-resistance to either doramectin or diazinon in those pyrethroid-resistant horn flies with target site mutations. If certain populations of horn flies that are resistant to pyrethroids are also more susceptible to diazinon than susceptible flies, then the mechanism for this form of negative cross-resistance would likely be metabolic rather than the *kdr* mutation for target site resistance. This information could be discerned in any fly population by a combination of the PCR assays and the time to 50% mortality bioassays reported here. We would need to know the role of *kdr* and metabolic mechanisms in the resistance levels for populations of flies in order to select an optimal resistance management strategy. Barros et al. (1999) reported that an annual rotation between pyrethroid and OP tags was not an effective strategy for maintaining susceptibility of flies at two sites in Louisiana. We recently conducted a retrospective study on the genotype of the flies at one of those sites and found that there was a high incidence of the resistant *kdr* allele (Guerrero et al., 2002). We observed that the incidence of the resistant *kdr* alleles increased each year that the pyrethroid was used, but that there was no observable trend when the OP was used; that is, the incidence of the resistant *kdr* allele did not change with the use of the OPs. We proposed that if the *kdr* target site resistance is fixed in a horn fly population, then a strategy other than alternating with OP treatments might be required to reverse pyrethroid resistance. We did note that the incidence of resistant *kdr* alleles reduced from fall to spring each year over the 7 years study. These data supported reports that the *kdr* mutation is associated with fitness costs, particularly in reduced biotic potential of resistant flies (Scott et al., 1997). Thus, for the target site-resistant populations, studies to determine the causation of fitness costs may allow us to develop strategies to exploit the mechanisms, and regain balance of susceptible genotypes.

Although there were limitations, we also were able to examine some of the interactions between specific genotype combinations of the *kdr* and *superkdr* mutations in relation to their contribution to survival of pesticide exposure. It is more difficult to ascertain the effects of the *superkdr* genotype on pyrethroid resistance because the *superkdr* mutation has not been found in isolation from the *kdr* mutation. Further, the only genotypes we were able to statistically compare were those that had a significant number of flies that were alive or dead in the assay, and that number was influenced by the level of pyrethroid resistance at the three locations. The resistance of an individual is determined by its genotype and the differing resistance levels of the three horn fly populations were evidenced by the weeks of control by ear tag and the times and genotype distributions of the survivors in the 50% mortality bioassay. For example, flies were controlled at the St. Joseph and Winnsboro locations for 3 and 0 weeks, respectively, while the estimated time for 50% mortality at St. Joseph for permethrin and λ-cyhalothrin was 17 min for both chemicals, and at Winnsboro, the time was 35 min for permethrin and 40 min for λ-cyhalothrin. Table 8 shows that the S allele frequency of the St. Joseph and Winnsboro populations was 27.01 and 14.38%, respectively (Table 8). Thus, all measures of pyrethroid susceptibility consistently showed the St. Joseph population of horn flies is more susceptible to pyrethroids than the Winnsboro population.

When possible interactions of the different genotypes of *kdr* and *superkdr* were compared, we found that there were no associations with the response to the OP and doramectin, but that there were certain comparisons that were statistically different in the response to pyrethroids (Table 9). First, we found that SS–SS was more susceptible to the pyrethroids than the other genotypes combined at Red River. We also were able to use the St. Joseph data, which had sufficient numbers of individuals with only the wild type susceptible *superkdr* allele, to demonstrate the SS–SR genotype was more susceptible to the pyrethroids than the SS–RR genotype. These data help to confirm that, in the absence of the resistant form of the *superkdr* allele, the heterozygote *kdr* genotype is intermediate in susceptibility to pyrethroids between the homozygous susceptible and the homozygous-resistant

genotypes at the *kdr* locus. Also at the St. Joseph location, we had sufficient individuals to show that flies with the SR–SR genotype was significantly more susceptible to pyrethroids than SR–RR individuals. Thus, the R allele dosage at the *kdr* locus is related to pyrethroid resistance in either the presence or absence of a resistant *superkdr* allele.

A factor that might confound the analysis of the *superkdr* genotypes is the likely usage of alternate exons in the region of the sodium channel gene, which contains the *superkdr* locus. Several insect species, including *D. melanogaster* and *M. domestica*, utilize alternative splicing to produce variants of the sodium channel-coding region (Soderlund and Knipple, 2003; Lee et al., 2002). Although it is not known if *H. irritans* possesses the alternate exon strategy for producing sodium channel transcripts, remarkable similarity to *M. domestica* sodium channels at the intron position and amino acid sequence level have been reported (Guerrero et al., 1997; Jamroz et al., 1998). The site of the *superkdr* mutation is in a pair of mutually exclusive exons and since our PCR assay utilizes genomic DNA as template, we might not be detecting the functional genotype at the *superkdr* locus. If, as is the case for *M. domestica* (Lee et al., 2002), one of the pair of the mutually exclusive exons contains a stop codon, usage of this exon would likely result in a transcript which, if translated, would not result in a functional sodium channel. However, since the sequence remains in the genomic DNA, it would be detected by the PCR. If this non-utilized exon retained the non-mutated susceptible Met codon, a susceptible allele would be indicated for the fly, even if the functional sodium channel possessed the mutated sequence coding for Thr. Thus, our data would over-report the frequency of the S *superkdr* allele in these populations. Nevertheless, the statistically significant association of increased resistance with increased *superkdr* R allele frequency was found in our data analysis. Alternate exon usage coupled with over-reporting of S alleles might explain the low levels of *superkdr* homozygous-resistant (RR) flies found in these wild populations (2-4%, Table 8), although Jamroz et al. (1998) utilized the identical PCR assay and reported 62% *superkdr* homozygous RR flies in the super-resistant colony which had been treated weekly with pyrethroid for 5 years prior to their study. The usage of alternate exons in *H. irritans* is being further explored by sequence analysis of gene regions surrounding the *superkdr* locus.

The Winnsboro population had a large percentage of homozygous-resistant (RR) *kdr* alleles, which allowed the testing of the effect of *superkdr* allele dosage on pyrethroid resistance. That analysis led to the statistically significant finding that for the flies possessing the homozygous-resistant *kdr* genotype, individuals with the homozygous susceptible (SS) *superkdr* genotype were more susceptible to pyrethroids than flies with SR–*superkdr*. Thus, SR–RR individuals are more resistant to pyrethroids than the SS–RR individuals. Unfortunately, the SR–SS versus SR–SR comparison cannot be made since the *superkdr* mutation has not been found in the absence of the *kdr* mutation. Lee et al. (1999) postulated that the *superkdr* mutation was a second-site mutation in populations with the *kdr* mutation and that the *superkdr* mutation permitted a higher expression of resistance levels to pyrethroids. Our study supports those two ideas, as (1) we did not find any individuals, who possessed the *superkdr* mutation without a corresponding *kdr* mutation and (2) possession of resistant *superkdr* alleles conferred a higher level of pyrethroid resistance than possession of the susceptible *superkdr* alleles. Given the differential responses of the each genotype, we should be able to establish discriminating doses or time tests to screen for different genotypes, and such tests could lead to better recommendations for resistance management.

Acknowledgments

We thank Mary Brumley and Melinda May for assistance with the PCR assays, Glenn Oremus for assistance during the bioassays, and Andrew Li and Andy Chen for critical review of the manuscript. Published with approval of the Director of Louisiana Agricultural Experiment Station as Manuscript No. 04-26-0089.

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